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John B. Harley *et al.*

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Steven L. Highlander

INVENTORS' DECLARATION UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

We, the undersigned, do declare that:

1. We are the John B. Harley, Kenneth M. Kaufman and Judith A. James named as inventors on the above-captioned application. John B. Harley is a citizen of the United States residing at 439 NW 20th Street, Oklahoma City, OK. Kenneth M. Kaufman is a

2. We are also the John B. Harley, Kenneth M. Kaufman and Judith A. James named as authors on the attached manuscript entitled "An altered immune response to Epstein-Barr virus suggests a link to systemic lupus erythematosus," by McClain *et al.* This manuscript has been submitted for publication and in presently under review.

3. In the manuscript, we describe studies designed to test whether the immune response to EBV acts as a risk factor for pediatric SLE and assesses the extent that the fine specificity of the anti-EBNA-1 response in SLE can be distinguished from normal control humoral responses. Pediatric lupus patient and healthy, matched control sera were tested to define the fine specificity of their anti-EBNA-1 humoral immune response utilizing a modified ELISA assay against the maximally overlapping octapeptides of EBNA-1.

4. Thirty-six SLE patients and their matched EBV positive controls were demonstrated to produce anti-EBV-VCA antibodies. All 36 SLE patients produced antibodies against a 70 kD band in the EBV infected lysates (from B95-8 or Jijoye), as well as by whole EBNA-1 ELISAs. Of the 36 sera from EBV-positive controls (matched on age, sex, and race), 11 (31%) did not produce detectable antibodies which recognized EBNA-1 from either the B95-8 or the Jijoye cell lines. Anti-EBNA-1 antibodies are therefore associated with SLE (OR=30.4, χ^2=13.3, p<0.005, CI 95% 1.7 to 544).

5. To explore differences in the humoral responses between cases and controls, expressed fragments containing the N-terminal (amino acids 1-89), middle (amino acids 90-330),

and carboxyl terminal (amino acids 331-641) regions of EBNA-1 were tested. Twenty pediatric SLE patient sera and 20 sera from their matched, EBV-positive controls were randomly selected. Compared to the control sera, the SLE patients had relatively greater than average binding to the N-terminal fragment (containing the glycine-arginine rich segment) (mean OD=0.451 versus 0.268) and to the carboxy terminal fragment (OD=0.844 versus 0.296). Controls (OD=0.779) have higher average binding to the glycine-alanine rich middle segment (containing the multiple glycine-alanine repeats) when compared to the SLE patients (OD=0.396). All pairwise case-control comparisons are significant by student's T test ($p<0.001$).

6. The 20 lupus patient sera tested bound many different overlapping octapeptides of the EBNA-1 protein, while normal EBV-negative individuals do not. Binding of a representative SLE patient serum shows the many epitopes typically bound in the amino and carboxyl terminal regions of EBNA-1 in most of these pediatric SLE sera. In contrast, the anti-EBV-VCA positive normal control serum illustrates the stark differences usually observed between cases and controls, which is also demonstrated by the mean binding of SLE and their control sera. Anti-EBV-VCA negative control sera showed no significant reactivity (average background binding OD=0.118).

7. Among epitopes selectively bound by the SLE pediatric sera, amino acids 40-53 (GRGRGRGRGRGGR), known as the (GR_X) region, and amino acids 398-404 (PPPGRRP) are commonly targeted, although this latter epitope just fails to achieve statistical significance when all SLE patients are averaged (binding is 1.98 standard

deviations above the normal mean). However, all of these anti-PPPGRRP positive sera also bind Sm in the standard solid phase assay (data not shown). Some variation in the binding of individual SLE patient sera to these octapeptides does occur. For instance, about 67% of the SLE patient samples bind PPPGRRP. Tested pediatric SLE samples, however, do not significantly bind (>2 S.D.), the regions comprising the (GA_X) repeats that are the primary target of the normal humoral immune response.

8. In contrast the normal, EBV-positive pediatric controls reveal an entirely different binding pattern to the overlapping octapeptides of EBNA-1. Normal children and teenagers make antibodies primarily against two epitopes of the EBNA-1 protein (both of which are present in the middle recombinant EBNA-1 fragment). These epitopes consist of amino acids 101-113 (GGAGAGGGAGAGG) and amino acids 140-155 (GGAGAGGGAGAGGGAG), neither of which are recognized by the pediatric SLE patient sera. Furthermore, a group of five EBV-positive polymyositis patients show a nearly identical, limited response to the octapeptides of EBNA-1 as that seen in normal EBV-positive controls (data not shown). Unlike the SLE pediatric sera tested, non-SLE but EBV-positive individuals have a nearly uniform, almost monotonously replicated binding pattern, as is demonstrated by the close similarity between the representative example and mean binding. In fact, the mean binding pattern generally observed in the pediatric controls is different and virtually mutually exclusive from the pattern generated by the SLE pediatric patient sera. This result strongly suggests that the molecular and cellular immunoregulatory events that lead to anti-EBNA-1 antibodies are significantly

different in the pediatric SLE patients than they are in their normal pediatric matched controls.

9. CMV-IE was chosen as a control antigen since it is a DNA binding protein found in another life-long, common herpes virus infection and since CMV-IE is known to induce an antibody response in normal CMV-infected individuals. Both SLE patients and normal individuals recognize multiple, almost identical epitopes of CMV-IE. In contrast to the distinct qualitative differences between SLE and normal immune responses to EBNA-1, the SLE sera bind the same epitopes of the CMV-IE octapeptides less avidly than control sera.

10. We hereby declare that all statements made of our own knowledge are true and all statements made on information are believed to be true and further that the statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issued thereon.

Date	John B. Harley
Date	Kenneth M. Kaufman
Date	Judith A. James

An altered immune response to Epstein-Barr virus suggests a link to systemic lupus erythematosus

Micah T. McClain[1,2], Monica Y. Kirby[1], Kenneth M. Kaufman[1,2,3], John B. Harley[1,2,3], and *Judith A. James[1,2]

Oklahoma Medical Research Foundation[1],
University of Oklahoma Health Sciences Center[2], and
US Department of Veterans Affairs Medical Center[3], Oklahoma City, OK 73104

*Corresponding Author:

Judith A. James, M.D., Ph.D.
Arthritis and Immunology
Oklahoma Medical Research Foundation
825 N.E. 13th Street
Oklahoma City, Oklahoma 73104
Phone: 405-271-4987
Fax: 405-271-7063
E-mail: jamesj@omrf.ouhsc.edu

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Running Title: Anti-EBNA-1 antibodies in SLE

Abstract

Objective: An evolving body of biomedical research is continually providing evidence that host immune responses to pathogenic organisms can act as the nidus for autoimmunity. One such potential agent, the Epstein-Barr virus, has been strongly linked to systemic lupus erythematosus through both association studies and experimental data demonstrating the apparent origin of SLE anti-Sm autoimmunity from humoral immune responses to EBNA-1. This paper tests whether the immune response to EBV acts as a risk factor for pediatric SLE and assesses the extent that the fine specificity of the anti-EBNA-1 response in SLE can be distinguished from normal control humoral responses.

Methods: Pediatric lupus patient and healthy, matched control sera were tested to define the fine specificity of their anti-EBNA-1 humoral immune response utilizing a modified ELISA assay against the maximally overlapping octapeptides of EBNA-1.

Results: All 36 pediatric SLE patient sera tested recognize EBNA-1, while 11 of 36 matched, EBV-positive controls do not target EBNA-1 ($p<0.005$). Epitope mapping reveals that the humoral anti-EBNA-1 response in pediatric SLE is completely distinct from that seen in matched normal individuals; the SLE sera bind many epitopes, each of which is distinct from the two major regions recognized by normal control sera. Meanwhile, no differences were seen in the SLE response to other Herpes viruses, and binding of SLE sera to sequential epitopes from the Cytomegalovirus-Immediate Early Antigen is qualitatively indistinguishable from control sera.

Conclusion: This altered humoral immune response to EBNA-1 may be a key SLE susceptibility factor.

Introduction

Systemic lupus erythematosus (SLE) is a chronic, systemic, idiopathic, clinically heterogeneous autoimmune disease. Nearly all patients have high titers of serum autoantibodies (1-2) that usually precede clinical SLE onset by many years (3). Many potential environmental factors have been evaluated, though consensus supporting a particular unifying susceptibility factor has not been obtained (4).

Epstein-Barr virus (EBV) infection is one of the environmental risk factors most closely associated with SLE. Virtually all pediatric SLE patients have serologic evidence of EBV infection, while only 70% of pediatric controls are seropositive (OR=49.9, CI-95%= 9.3 to 1025, $p<0.0001$), a result that is confirmed at the level of recovered EBV DNA (5-6) and in antinuclear antibody-positive adult SLE patients (7).

EBV appears well suited to initiate autoimmune disease. EBV infects B cells and promotes dysregulated polyclonal B cell activation and autoantibody production (8-11). EBV latency provides life-long antigenic challenge (8). EBV also produces viral proteins that prevent apoptosis (through a bcl-2 homologue) and have IL-10-like activity (8-9).

EBNA-1 exhibits a very unusual antigen presentation, as full-length EBNA-1 seems to be readily expressed through the MHC class II pathway, whereas defective, truncated ribosomal products of EBNA-1 seem to be required for presentation of EBNA-1 peptides through the Class I pathway (12-15). Approximately 95% of normal, EBV infected adults make antibodies directed against EBNA-1, which are dominated by antibodies binding the repetitive glycine-alanine rich central region of EBNA-1 (16).

The first anti-Sm autoantibodies to appear in SLE patient sera bind a structure (PPPGMRPP) that cross-reacts with a very similar structure in EBNA-1 (PPPGRRP) (17). This EBNA-1 structure is a common, although transient, antigenic epitope in recent EBV infection resulting in mononucleosis (11). Immunization with PPPGMRPP of Sm B' or PPPGRRP of EBNA-1 on a branching polylysine backbone induces anti-Sm autoantibodies. This response then expands through epitope spreading to include autoantibodies against other spliceosomal proteins and double-stranded DNA, and induces an SLE-like illness in experimental animals (18-20). These observations support the hypothesis that anti-Sm autoantibodies may arise from anti-EBNA-1 antibodies. Another immune structural relationship between the autoantigen Sm D1 and

the GR_x repeat of EBNA-1 is described, but as yet has no known relationship to the original generation of anti-Sm autoimmunity in man (17,21-25).

Since EBV infection approaches being ubiquitous in man and SLE is uncommon, other factors beyond simple EBV infection must be risk factors for SLE. We suspect that features of the host immune response against EBV are such an explanatory risk factor and herein report results supporting this hypothesis, specifically with regard to anti-EBNA-1 antibodies.

Methods

Sera

Patients satisfying the ACR criteria for SLE (26) and under 20 years of age were consented and enrolled to this IRB-approved protocol. Patients were asked to provide an age ($\pm$ 2 years and <20 years), sex, and race matched control. Preferably, controls were siblings, then cousins or friends. (Forty-four percent of controls were related to their matched case.) Demographic information includes age (15.2 vs. 15.7 years), race (pairs included 45% European-American, 35% African-American, 10% Hispanic, 5% American Indian, and 5% Eastern Indian), geographical location (all from Oklahoma and Kansas), and sex (95% female). For these experiments only controls with evidence of EBV seroconversion were used. Additionally, sera from a group of five non-matched adult patients meeting diagnostic criteria for polymyositis were utilized as another rheumatic disease control group.

Epstein-Barr Viral Capsid Antigen ELISA assays

Antiviral assays (anti-EBV-viral capsid antigen (VCA) and anti-Cytomegalovirus Immediate-Early antigen (CMV-IE) from Wampole Laboratories, Cranberry, NJ) were conducted as outlined by the manufacturer. Results and analyses are presented as units of the international standardized ratio (ISR), which are designed to detect seroconversion. Since the ISR is semi-quantitative and is not linear with optical density, higher levels of antibody are relatively underestimated.

Production of EBV lysates

Cell lysates from Ramos (no EBV infection), B95-8 (EBNA-1 expressing EBV strain-1), and Jijoye (an EBNA-1 expressing EBV strain-2) were generated. Approximately $1.2x10^8$ cells were pelleted at 1500 rpms for 5 mins. The cells were then washed with 0.01M PBS, pH 7.2. After two more washes the cells were resuspended in cell lysis buffer (0.5% Nonidet P-40, 0.15M NaCl, 0.05M Tris-base, 5mM EDTA, pH 8.0) at $1x10^8$ cells/mL. The cells were then sonicated for 80 sec (20 sec bursts with 20 sec icing intervals). Cell lysates were solubilized with an equal volume of sample buffer (0.0625M Tris, 2% SDS, pH 6.8), boiled for 2 minutes, and centrifuged at 14,000 rpms for 10 minutes before immunoblotting.

Immunoblot analysis of viral lysates

Cell extracts of B95-8, Jijoye, and Ramos cell lines were electrophoresed on 12.5% polyacrylamide gels containing 1% SDS (in 0.15 M Tris HCl, pH 8.8) under reducing conditions. The proteins were transferred onto nitrocellulose and sera (1:100 dilutions) were tested for binding to lysate proteins, as previously described (27). Binding of antibodies to proteins in the extract was detected with alkaline-phosphatase-conjugated anti-human IgG diluted to 1:5000 (Jackson Immunoresearch Laboratories, West Grove, PA), with an anti-EBNA-1 monoclonal antibody ab8329 (Abcam, Cambridge, MA) as positive control.

EBNA-1 fragments

EBNA-1 DNA was amplified from B95-8 cells using 5' primers containing an *Eco*RI restriction site and 3' primers containing a *Hind*III site. DNA encoding the N-terminus of EBNA-1 (amino acids (aa) 1-89), the middle region (aa 90-330) and the C-terminus (aa 331-641) were amplified (Figure 1). Both the 5' and middle region polymerase chain reaction (PCR) products contained an in-frame stop codon. The N-terminal region of EBNA-1 was amplified with forward 5' TTGAATTCATGTCTGACGAGGGGC 3' and reverse 5' TTAAGCTTATGTTCCACCGTGGGTCCC 3' primers. The middle EBNA1 fragment was amplified with forward 5' TTGAATTCGGAGCAGGAGCA GGAGCG 3' and reverse 5' TTAAGCTTAACCCCGGCCTCCACCTCC 3'. The C-terminal Fragment was amplified with forward 5' TTGAATTCCGAGGAGGCAGTGGAGGC 3' and reverse 5' TTAAGCTTCACTCCTGCCCTTCCTC 3'. PCR reactions were performed in 50 μL total

volume using 100 ng genomic B95-8 DNA, 10 pmol of the EBNA-1 specific primers, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 2 units Taq polymerase. PCR was performed at 94°C for 1 min, 50°C for 2 min, and 72° C for 3 min for a total of 30 cycles, followed by 10 min at 72°C. PCR products were gel purified and cloned into the pCR2.1 (Invitrogen, Carlsbad, CA). The fidelity of the PCR products was verified by sequencing. Cloned EBNA-1 fragments where excised with *Eco*RI and *Hind*III and cloned into the pMal-C2 expression vector (New England Biolabs, Beverly, MA). Recombinant fragments were expressed following the manufacturer's instructions.

Standard solid-phase ELISAs were used to measure the antibody in human SLE and control sera. Two μg of antigen (EBNA-1 fragmented fusion proteins) were coated per well in 96-well polystyrene microtiter dishes, following our published protocol (17). Briefly, antibodies from test serum samples (at 1:100 and 1:1000 dilutions) were analyzed for reactivity with bound EBNA-1 fragements as described above. Plates were standardized across assays by normalizing a positive control to an optical density of 1.0 at an absorbance of 410 nm.

Solid Phase Peptide Synthesis and Anti-Peptide Assays

Overlapping octapeptides of EBNA-1 and CMV-IE were sequentially synthesized at the ends of radiation derivatized polyethylene pins arranged in the format of a 96-well microtiter plate, as described previously (11). Duplicate octapeptides (especially important in the glycine-alanine rich region (aa 97-321)) were deleted. The unique octapeptides from EBNA-1 (aa 1-96, 101-106, 110-120, 137-151, 202, & 321-634) were synthesized. All 484 overlapping octapeptides of CMV-IE were similarly constructed. In addition, positive control pins for both sets were synthesized from a known reactive sequence of the Sm B' protein (PPPGMRPP) and used with previously characterized positive and negative sera as standards. Serum samples from SLE patients, EBV-positive unaffected controls, EBV-positive polymyositis patients, and EBV-negative normals were tested for binding with the maximally overlapping octapeptides of EBNA-1 and of CMV-IE by a solid phase assay, as previously described (11).

Results

Anti-EBNA-1 antibodies are more common in SLE patients

All 36 SLE patients and their matched EBV positive controls were demonstrated to produce anti-EBV-VCA antibodies. All 36 SLE patients produced antibodies against a 70 kD band in the EBV infected lysates (from B95-8 or Jijoye) in immunoblot assays (Figure 1). This 70kD band has the same mobility in polyacrylamide gel electrophoresis as EBNA-1. Further, this band was recognized by an EBNA-1 monoclonal antibody (ab8329, Abcam Ltd., Cambridge, MA) and this band was not detected in a non-EBV infected cell line (Ramos) by any serum tested. (Data are not presented.)

Of the 36 sera from EBV-positive controls (matched on age, sex, and race), 11 (31%) did not produce detectable antibodies which recognized EBNA-1 from either the B95-8 or the Jijoye cell lines. Anti-EBNA-1 antibodies are therefore associated with SLE (OR=30.4, χ^2=13.3, $p<0.005$, CI 95% 1.7 to 544). No significant differences in anti-EBNA-1 recognition specificity between the two strains of EBV (B95-8 and Jijoye) were observed in either cases or controls.

Lupus patients respond differently to recombinant EBNA-1 fragments

To explore differences in the humoral responses between cases and controls we tested expressed fragments containing the N-terminal (amino acids 1-89), middle (amino acids 90-330), and carboxyl terminal (amino acids 331-641) regions of EBNA-1 (Figure 2). Twenty pediatric SLE patient sera and 20 sera from their matched, EBV-positive controls were randomly selected. Compared to the control sera the SLE patients had relatively greater average binding to the N-terminal fragment (containing the glycine-arginine rich segment) (mean OD=0.451 versus 0.268) and to the carboxy terminal fragment (OD=0.844 versus 0.296) (Figure 2). On the other hand, controls (OD=0.779) have higher average binding to the glycine-alanine rich middle segment (containing the multiple glycine-alanine repeats) when compared to the SLE patients (OD=0.396). All pairwise case-control comparisons are significant by student's T test ($p<0.001$).

SLE patients recognize unique epitopes of EBNA-1

The 20 lupus patient sera tested bound many different overlapping octapeptides of the EBNA-1 protein, while normal EBV-negative individuals do not. Binding of a representative SLE patient serum shows the many epitopes typically bound in the amino and carboxyl terminal

regions of EBNA-1 in most of these pediatric SLE sera (Figure 3). In contrast, the anti-EBV-VCA positive normal control serum illustrates the stark differences usually observed between cases and controls (Figure 3B), which is also demonstrated by the mean binding of SLE and their control sera (Figure 3C & 3D). Anti-EBV-VCA negative control sera showed no significant reactivity (average background binding OD=0.118). Among epitopes selectively bound by the SLE pediatric sera, amino acids 40-53 (GRGRGRGRGRGGR), known as the (GR_X) region, and amino acids 398-404 (PPPGRRP) are commonly targeted, although this latter epitope just fails to achieve statistical significance when all SLE patients are averaged (binding is 1.98 standard deviations above the normal mean). However, all of these anti-PPPGRRP positive sera also bind Sm in the standard solid phase assay (data not shown). Some variation in the binding of individual SLE patient sera to these octapeptides does occur. For instance, about 67% of the SLE patient samples bind PPPGRRP. Tested pediatric SLE samples, however, do not significantly bind (>2 S.D.), the regions comprising the (GA_X) repeats that are the primary target of the normal humoral immune response.

In contrast the normal, EBV-positive pediatric controls reveal an entirely different binding pattern to the overlapping octapeptides of EBNA-1 (Figure 3). Normal children and teenagers make antibodies primarily against two epitopes of the EBNA-1 protein (both of which are present in the middle recombinant EBNA-1 fragment). These epitopes consist of amino acids 101-113 (GGAGAGGGAGAGG) and amino acids 140-155 (GGAGAGGGAGAGGGAG), neither of which are recognized by the pediatric SLE patient sera. Furthermore, a group of five EBV-positive polymyositis patients show a nearly identical, limited response to the octapeptides of EBNA-1 as that seen in normal EBV-positive controls (data not shown). Unlike the SLE pediatric sera tested, non-SLE but EBV-positive individuals have a nearly uniform, almost monotonously replicated binding pattern, as is demonstrated by the close similarity between the representative example and mean binding (Figure 3). In fact, the mean binding pattern generally observed in the pediatric controls is different and virtually mutually exclusive from the pattern generated by the SLE pediatric patient sera. This result strongly suggests that the molecular and cellular immunoregulatory events that lead to anti-EBNA-1 antibodies are significantly different in the pediatric SLE patients than they are in their normal pediatric matched controls.

CMV-IE was chosen as a control antigen since it is a DNA binding protein found in another life-long, common herpes virus infection and since CMV-IE is known to induce an antibody response in normal CMV-infected individuals. Both SLE patients and normal individuals recognize multiple, almost identical epitopes of CMV-IE (Figure 4). In contrast to the distinct qualitative differences between SLE and normal immune responses to EBNA-1, the SLE sera bind the same epitopes of the CMV-IE octapeptides less avidly than control sera (Figure 4).

Discussion

A role for EBV in SLE has been suspected for over 30 years (28-33). Indeed, the earliest published study from 1969 uses SLE sera as controls and shows possible association of anti-EBV serology (as measured by precipitins) with SLE (28). Efforts to establish whether SLE is associated with positive anti-EBV serology have met with mixed results. Authors in the 1970s rejected this hypothesis, which became the scientific dogma (32), until recently.

EBV infection has long been known to induce the production of autoantibodies (22-23, 34). In fact, many infectious mononucleosis patients are demonstrated to temporarily produce antibodies targeting the Sm autoantigen, normally considered specific for SLE (11). Additionally, the recent use of more reliable technologies to detect antibody binding establishes a strong association between EBV infection and SLE (5-7). However, little data detailing specific anti-EBV antibody profiles in actual lupus patients has previously been available. In this study, lupus patients are demonstrated to make statistically higher amounts of antibody against the fragments encompassing the amino and carboxyl ends of EBNA-1, while normal EBV-positive controls actually make significantly higher levels of antibody than lupus patients against the middle fragment. However, this is not merely the difference between a simple response (in normals) and one that is more complex (in SLE). These responses are distinct from one another. Normal individuals make antibodies against two specific major epitopes of EBNA-1, contained in the middle portion of the protein. Pediatric lupus patient sera, while recognizing many more epitopes, seem to conspicuously avoid the two regions which are antigenic in normals (Figure 3). Finally, analysis of the antigenic epitopes of CMV IE in these individuals demonstrates the specificity of this dysregulated immune response for EBV, since both normals and SLE patients show similar reactivities to CMV IE peptides (Figure 4).

How can we explain two such disparate immune responses? In lupus patients, specific antigenic cross-reactivity of EBV proteins with spliceosomal autoantigens, such as Sm B' and Sm D1, identify molecular relationships potentially responsible for the generation of autoimmunity (11, 18, 22-25), which is bolstered by work in animal models and the demonstration of B cell epitope spreading (18-21). Sabbatini et al recognized that while a few normal individuals make antibodies against the (GR_X) rich portion of EBNA-1, these antibodies do not cross-react with a (GR_X) rich portion of Sm D1 (22). The anti-(GR_X) from lupus patients, however, strongly cross-reacts with the spliceosomal autoantigen. One possibility is that lupus patients are deficient in the ability to prevent the loss of tolerance to self-antigens upon exposure to a potentially cross-reactive agent such as EBV. In normal individuals, immunity against cross-reactive epitopes of pathogens could be suppressed or is otherwise unproductive, resulting in one pattern of anti-EBNA immunity as described herein. Indeed, this theory is bolstered by data demonstrating that Infectious Mononucleosis patients temporarily produce antibodies which target a carboxyl-terminal epitope of EBNA-1 (aa 398-404, also recognized by 67% of SLE patients in our current study) which cross-reacts with the lupus autoantigen Sm B' (11). These (auto)antibodies are cleared by about 6 months post-infection in normal individuals with mononucleosis (11), whereas they persist in many lupus patients (as demonstrated herein), even into and throughout adulthood (17) . Furthermore, deficits in anti-EBV immunity such as disrupted antibody-dependent cellular cytotoxicity of EBV-infected cells and defective T cell suppression of EBV-induced B cell proliferation have been demonstrated in SLE patients (32-33). In SLE, then, known defective immunity and tolerance mechanisms (35-38) provide an environment which permits a different pathway of anti-EBNA-1 immunity (involving cross-reactive regions), as we have shown. Whether this alternate path towards anti-viral immunity contributes to lupus etiology or pathogenesis is not entirely clear. Certainly, potential mechanisms for induction of autoimmunity by EBV have been demonstrated (9-12, 22-25, 34-36), and the differences in normal and lupus immune responses outlined here are consistent with the possibility that autoimmunity could originate in the details of the structural and molecular relationships of the anti-EBNA-1 humoral immune response.

Anti-EBNA-1 in SLE may therefore be another example of molecular mimicry in autoimmunity, with the pathogenic autoantibodies emerging as cross-reactive specificities

generated in the host response against an infection. In man, there are many such examples, of which the best may be the recent demonstration that Sydenham's chorea, an expression of rheumatic fever, appears to be caused by antibodies originally directed against *Streptococcus pyogenes* that cross react with N-acetyl-β-D-glucosamine in the central nervous system (39).

We have tested whether the demonstrated association between SLE and EBV originates with the anti-EBV humoral immune responses. Since previous data suggest a molecular mimicry mechanism between EBNA-1 and Sm B' (11,22-25,34), EBNA-1 was the obvious focus for our experimental approach. Now, we further substantiate these ideas by demonstrating that pediatric SLE patients and matched normal individuals make distinct antibody responses against EBNA-1 and that anti-EBNA-1 is associated with SLE. In addition, these EBNA-1 data are in agreement with the previously reported three epitopes of EBNA-1 reactive in SLE but not in normals (23), done with a less discriminating technical approach. That EBNA-1 has a very unusual antigen presentation (12-15) offers many avenues to explore the pathophysiology and systemic autoimmunity of SLE. Certainly, potential mechanisms for induction of autoimmunity by EBV have been demonstrated, and the differences in normal and lupus immune responses outlined here are consistent with the possibility that those mechanisms play a role in the initiation or perpetuation of SLE.

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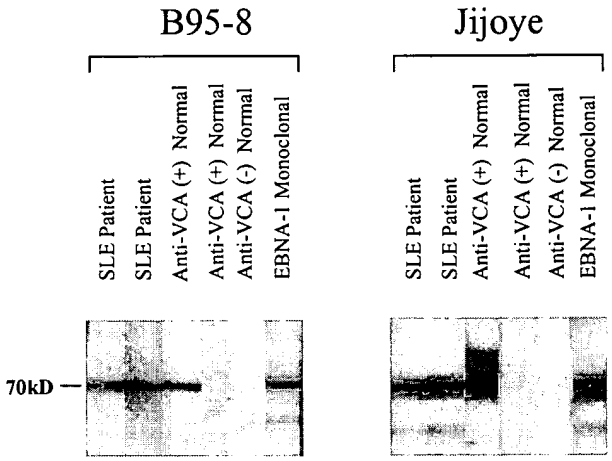
Figure Legends

Figure 1. Anti-EBNA-1 detected by immunoblot of EBV infected cell lysates. The EBNA-1 (70 kD band) is shown. Binding of representative SLE patients, EBV seropositive controls, and EBV seronegative normals are presented.

Figure 2. A) Map of the recombinant EBNA-1 fragments. Shown are the three constructs and their starting and ending amino acid numbers. Also depicted are the locations of major SLE and normal epitopes on the EBNA-1 peptides. B) Binding of SLE patient and EBV-positive control sera to the recombinant EBNA-1 fragments. SLE patient sera (black) exhibit higher levels of binding by standard ELISA to the amino terminal (n-term) and carboxyl-terminal (c-term) fragments than do controls (grey). Controls, however, exhibit stronger binding to the middle fragment.

Figure 3. Patient and control humoral reactivity to EBNA-1 peptide sequences. Antibody binding to the overlapping octapeptides of EBNA-1 (with duplicated sequences removed) by (A) an SLE patient serum and by (B) an EBV positive normal serum are presented. Mean binding to octapeptides of EBNA-1 and. Standard deviations above the normal mean (of EBV-negative individuals) for all patient (Panel C) and control (Panel D) sera are indicated. The epitopes involved in the normal pediatric control anti-EBNA-1 response are highlighted in grey, and are distinct from those recognized by pediatric SLE patient sera.

Figure 4. Patient and control humoral reactivity to a commonly targeted cytomegalovirus protein. Mean binding to octapeptides of CMV-IE by 10 SLE patient sera (Panel A) and 10 matched control sera (Panel B) are presented. The epitopes identified by anti-CMV-IE antibodies from SLE sera and normal control sera are identical and are highlighted by gray bars.



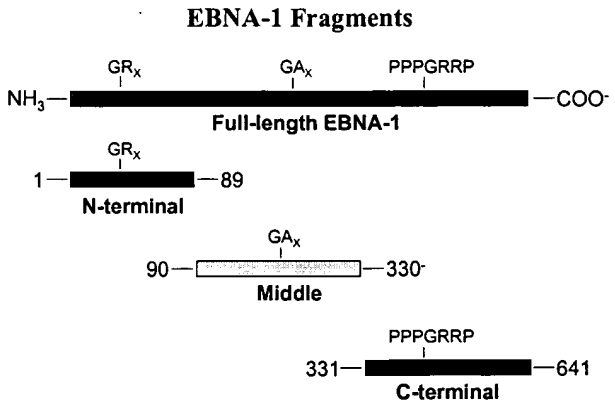
	SLE	Normal
EBNA-1 (+)	36	25
EBNA-1 (-)	0	11

$\chi^2 = 13.3$, $p<0.005$

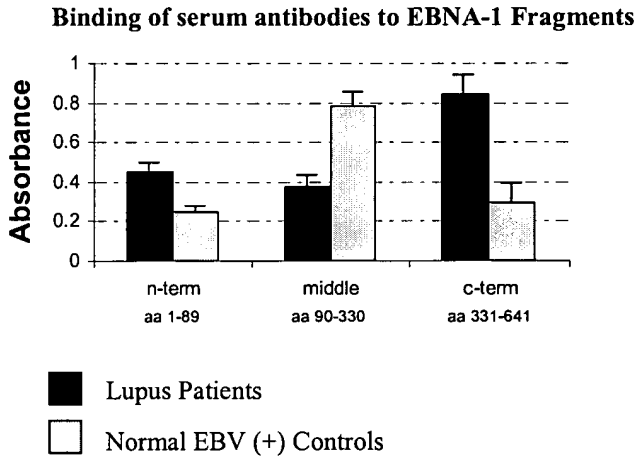
Odds Ratio = 30.4

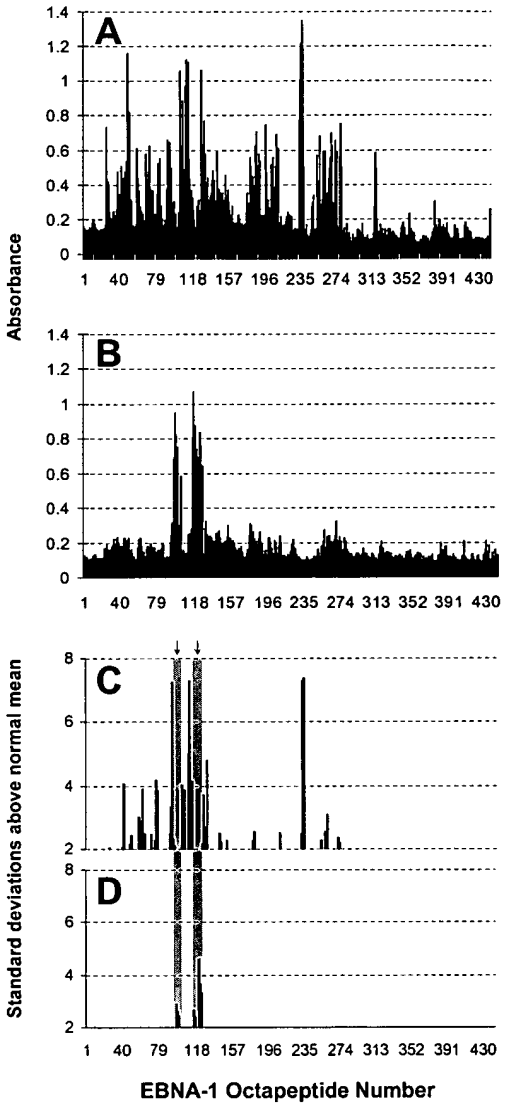
95% CI = 1.7 to 544

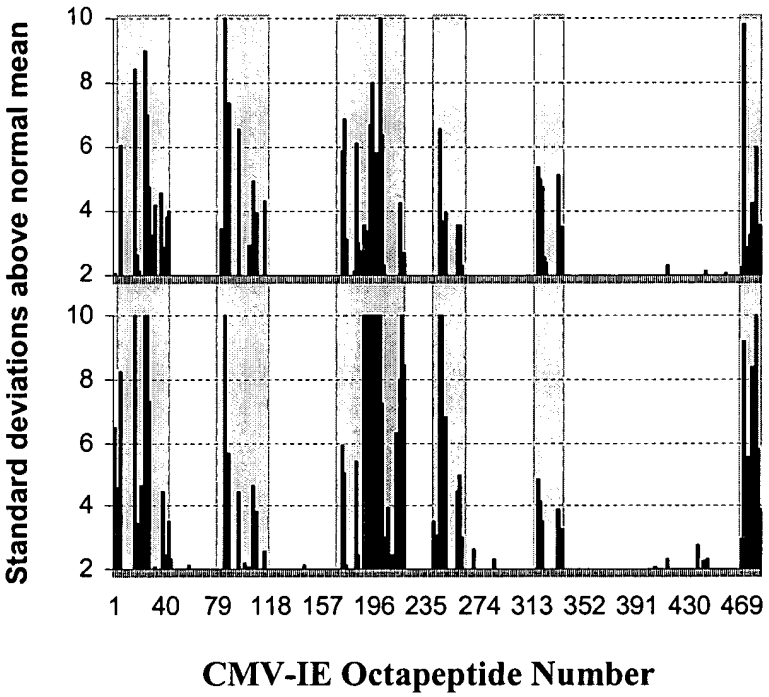
A


EBNA-1 Fragments
GRx
GAx
PPPGRRP
NH3—
—COO-
Full-length EBNA-1
GRx
1—
—89
N-terminal
GAx
90—
—330
Middle
PPPGRRP
331—
—641
C-terminal

B


Binding of serum antibodies to EBNA-1 Fragments
Absorbance
1
0.8
0.6
0.4
0.2
0
n-term
aa 1-89
middle
aa 90-330
c-term
aa 331-641
Lupus Patients
Normal EBV (+) Controls


A
B
C
D
Absorbance
Standard deviations above normal mean
1.4
1.2
1
0.8
0.6
0.4
0.2
0
8
6
4
2
1 40 79 118 157 196 235 274 313 352 391 430
EBNA-1 Octapeptide Number


Standard deviations above normal mean
10
8
6
4
2
10
8
6
4
2
1 40 79 118 157 196 235 274 313 352 391 430 469
CMV-IE Octapeptide Number